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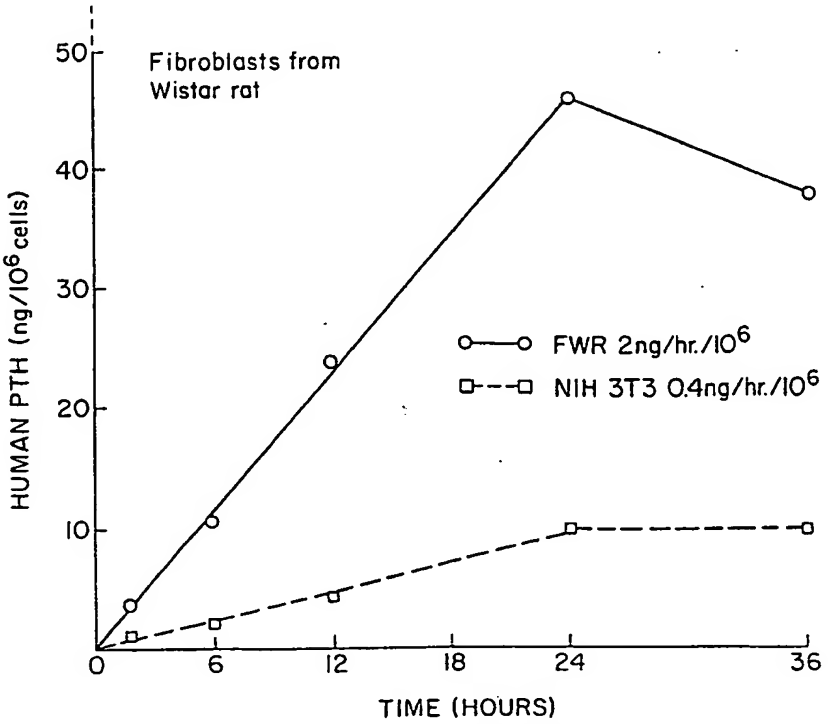

(57) Abstract

Fibroblasts transduced with genetic material encoding a polypeptide or protein of interest and, optionally, a selectable marker, as well as methods for making and using the transduced fibroblasts. Such fibroblasts are useful in delivering the encoded polypeptide or protein, such as an enzyme, a hormone or a drug, to an individual who has had a graft or implant of the transduced cells.

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TRANSDUCED FIBROBLASTS AND USES THEREFOR

Description

Background

In the embryo, the mesoderm is the middle of the three primary third germ layers, and lies between the ectoderm and the entoderm. It gives rise, during development, to the connective tissues, all body musculature, blood, cardiovascular and lymphatic systems, most of the urogenital system and the lining of the pericardial, pleural and peritoneal cavities. That portion of the mesoderm which produces the connective tissue, blood vessels and blood, the lymphatic system and the heart is referred to as the mesenchyme. One type of cells produced by mesenchymal cells is fibroblast cells, which are stellate or spindle-shaped cells with cytoplasmic processes. They are present in connective tissue and are capable of forming collagen fibers.

Fibroblasts, like other cells in the body, contain an entire complement of all genetic material. However, only a small percentage of the genes they contain are expressed at biologically functional levels. That is, most of the genes in fibroblasts are not expressed at all, or are expressed at such low levels that the polypeptides they encode are

produced in undetectable amounts or concentrations which are biologically nonfunctional or insignificant.

It is possible, using methods developed in recent years, to attain interspecies genetic recombination. Genes derived from different biological classes are able to replicate and be expressed in a selected microorganism. Therefore, it is possible to introduce into a microorganism genes specifying a metabolic or synthetic function (e.g., hormone synthesis, protein synthesis, nitrogen fixation) which is characteristic of other classes of organisms by linking the genes to a particular viral or plasmid replicon.

Since the late 1970s, progress has been made toward the development of general methods for introducing cloned DNA sequences into mammalian cells. At the present time, however, there is a need for an effective method of stably introducing genetic material into fibroblasts and enabling them to express genetic material which they do not normally express, or normally express at biologically insignificant levels.

Summary of the Invention

The invention described herein relates to genetically engineered mesenchymal or connective tissue cells and particularly to genetically engineered fibroblasts which express in biologically significant concentrations genetic material which is not normally expressed in significant concentrations in such cells. It also relates to methods of stably

introducing into fibroblasts such genetic material and methods of using the genetically engineered fibroblasts.

Fibroblasts of this invention have stably incorporated in them genetic material of interest and express the incorporated genetic material. This genetic material of interest is referred to herein as incorporated genetic material. The incorporated genetic material can be DNA or RNA which does not normally occur in fibroblasts; DNA or RNA which normally occurs in fibroblasts but is not expressed in them at levels which are biologically significant (i.e., levels sufficient to produce the normal physiological effects of the polypeptide it encodes); DNA or RNA which occurs in fibroblasts and has been modified so that it is expressed in fibroblasts; and any DNA or RNA which can be modified to be expressed in fibroblasts, alone or in any combination thereof. The incorporated genetic material expressed by fibroblasts of the present invention can include genetic material encoding a selectable marker, thus providing a means by which cells expressing the incorporated genetic material are identified and selected for. Fibroblasts containing incorporated genetic material are referred to as transduced fibroblasts.

In particular, retroviral vectors have been used to stably transduce fibroblasts with genetic material which includes genetic material encoding a polypeptide or protein of interest not normally expressed at biologically significant levels in fibroblasts. The genetic material introduced in this manner also

included genetic material encoding a dominant selectable marker. Genetic material including DNA encoding a polypeptide of interest and DNA encoding a dominant selectable marker has been introduced into cultured fibroblasts. Expression of these genes by the fibroblasts into which they have been incorporated (i.e., fibroblasts transduced by the use of retroviral vectors) has also been demonstrated.

A method of transplanting transduced fibroblasts which express the incorporated genetic material they contain is also a subject of the present invention. Transduced fibroblasts of the present invention are used for the constitutive delivery of polypeptides or proteins, useful in prevention and therapy or treatment, which are presently administered parenterally. They can be used in skin grafts and in glial cell or fibroblast implants which introduce DNA encoding a polypeptide or protein of interest into the central nervous system.

There are many advantages to fibroblasts of the present invention and the delivery system for a polypeptide or protein of interest which make them very useful. For example, a skin graft using fibroblasts which include incorporated genetic material encoding a polypeptide of interest (e.g., a hormone, enzyme, drug) synthesizes the encoded polypeptide and thus serves as a continuous delivery system for that polypeptide. In this way, the hormone or other polypeptide is diffused into the bloodstream of the individual receiving the skin graft.

An important advantage is that the genetically engineered fibroblasts of the present invention can be used to administer therapeutic proteins (e.g., hormones, enzymes, clotting factors) which are presently administered intravenously, intramuscularly or subcutaneously. In addition, there is no need for extensive (and often costly) purification of the polypeptide before it is administered to an individual, as is generally necessary with an isolated polypeptide (e.g., insulin). Fibroblasts modified according to the present invention produce the polypeptide hormone as it would normally be produced. In the case of insulin, for example, the genetically engineered fibroblasts produce insulin in the same form as that made in the pancreas.

Another advantage to the use of a graft having fibroblasts of the present invention is that by controlling the size of the graft, the amount of the polypeptide delivered to the body can be controlled. In addition, in the case of a skin graft, it can be excised if there is no longer a need for the polypeptide being produced. For example, if delivery of the polypeptide (hormone, enzyme, or drug) is necessary only for a specific period, the engineered graft can be removed when treatment is no longer needed.

Another important advantage of the delivery system of this invention is that because it is a continuous delivery system, the fact that polypeptide hormones have very short half lives is not a limitation. For example, the half life of human growth hormone (HGH) is approximately 19 minutes, of

parathyroid hormone, approximately 2½ to 5 minutes and, of native insulin (pure insulin), approximately 3 to 4 minutes.

Because genes can be introduced into fibroblasts using a retroviral vector, they can be "on" (subject to) the retroviral vector control; in such a case, the gene of interest is transcribed from a retroviral promoter. A promoter is a specific nucleotide sequence recognized by RNA polymerase molecules that start RNA synthesis. Alternatively, retroviral vectors having additional promoter elements (in addition to the promoter incorporated in the recombinant retrovirus) which are responsible for the transcription of the genetic material of interest, can be used. For example, a construct in which there is an additional promoter modulated by an external factor or cue can be used, making it possible to control the level of polypeptide being produced by the fibroblasts by activating that external factor or cue. For example, heat shock proteins are proteins encoded by genes in which the promoter is regulated by temperature. The promoter of the gene which encodes the metal-containing protein metallothionine is responsive to cadmium (Cd^{++}) ions. Incorporation of this promoter or another promoter influenced by external cues also makes it possible to regulate the production of the polypeptide by the engineered fibroblasts.

Brief Description of the Drawings

Figure 1 is a schematic representation of a wild type murine leukemia virus (retroviral) genome.

Figure 2 is a schematic representation of retroviral vectors, each having a recombinant genome, useful in the present invention. Figure 2a is pZIPNeo; Figure 2b is pLJ; Figure 2c is pWe; Figure 2d is pEm; and Figure 2e is pIp.

Figure 3 is a schematic representation of the construction of a recombinant retroviral vector, using the pLJ vector represented in Figure 2b and the human parathyroid hormone gene.

Figure 4 is a graph representing the quantity of human parathyroid hormone produced, in the designated time period, by transduced fibroblasts. The dashed line represents production by transduced NIH 3T3 cells on tissue culture plates. The solid line represents production by transduced fibroblasts derived from a rat and grown on tissue culture plates.

Figure 5 is a graph representing the quantity of human parathyroid hormone produced, in the designated time periods, in rats injected with either transduced fibroblasts alone (blackened circles) or transduced fibroblasts coated onto cyto 3 beads and grown to confluence.

Detailed Description of the Invention

Genetic material of interest has been incorporated into fibroblasts and expressed in the resulting genetically engineered fibroblasts. Genetic material incorporated into fibroblasts according to the method described can be DNA or RNA which does not normally occur in fibroblasts; DNA or RNA which normally occurs in fibroblasts but is not

expressed in them at levels which are biologically significant (levels sufficient to produce the normal physiological effects of the polypeptide it encodes); DNA or RNA which occurs in fibroblasts and has been modified so that it is expressed in such cells; and any DNA or RNA which can be modified to be expressed in fibroblasts, alone or in an combination thereof. This genetic material of interest is referred to herein as incorporated genetic material. Fibroblasts of the present invention express the incorporated genetic material. The incorporated genetic material (i.e., DNA or RNA) expressed by fibroblasts of the present invention is genetic material encoding a polypeptide or a protein of interest (genetic material of interest), alone or in combination with a gene encoding a selectable marker.

For example, genetic material encoding a hormone has been introduced into fibroblasts by exposing them to media that contains a virus having a recombinant genome (i.e., by infecting them). The media used is obtained by harvesting media in which producer cells (e.g., a Psi am or amphotropic producer) have been grown. That is, producer cells have been grown in tissue culture to a confluent density in Dulbecco's Modified Eagle's medium (DME) with 10% calf serum (CS) and penicillin and streptomycin. Fresh media is added and subsequently (e.g., approximately 12 hours later), the media is harvested. Approximately 10 ml of media is harvested from a 10 cm plate of confluent producer cells. The spent media (or viral stock) is filtered through a 0.45 micron Millipore filter to remove detached producer cells and is used

immediately to infect cells or is stored at -70° C. Media is removed from a subconfluent plate of fibroblasts (recipient fibroblasts) and quickly replaced with viral stock (e.g., 5 ml/10 cm. plate) containing 8 mcg/ml. of Polybrene (Aldrich). Subsequently (e.g., approximately 12 hours later), this is removed and replaced with fresh media. Thus, the media used is a viral supernatant. The recombinant genome of the infectious virus includes the genetic material of interest. The recombinant genome can also have genetic material encoding a dominant selectable marker.

Thus, fibroblasts are made which express a polypeptide not normally expressed by such cells at biologically significant levels and, optionally, a dominant selectable marker.

In particular, fibroblasts are exposed to media containing infectious virus produced in Psi am cells; the infectious virus contain a recombinant genome having the genetic material of interest. The recombinant genome in one instance includes genetic material encoding human parathyroid hormone (hPTH). Optionally, it can also include a gene encoding a dominant selectable marker (e.g., the neo gene which encodes neomycin resistance). As a result, the fibroblasts are transduced -- that is, the genetic material of interest (in this case, DNA encoding hPTH and, optionally, the neo gene) is stably introduced into the fibroblasts. The transduced fibroblasts express the encoded hPTH and, if the neo gene is present, express it, resulting in cells having the selectable trait.

Alternatively, fibroblasts are transduced as a result of exposure to media containing infectious virus in which the recombinant genome includes DNA encoding one or more of the following:

the receptor for low density lipoproteins;
human growth hormone;
the gene that confers resistance to histidonal;
human adenosine deaminase;
the receptor for interleuken 2;
human beta-globin;
human alpha-globin;
a mutant form of dihydrofolate reductase;
multidrug resistance;
glucose cerebrosidase from humans;
the E1A gene from adenovirus;
many different genes for HLA in humans;
human albumin;
human ornithine transcarbamalyase
beta-galactosidase from E. coli;
resistance to neomycin in E. coli;
human insulin; and
the envelope protein from Moloney murine leukemia virus.

Fibroblasts expressing the incorporated genetic material are grown to confluence in tissue culture vessels; removed from the culture vessel in which they were grown; and introduced into or applied to the body. They have been introduced into the intraperitoneal cavity, both alone and coated onto microcarrier beads (approximately 100 cells/bead)

having collagen-covered surfaces. Alternatively, they can be applied as a component of a skin graft, using a method such as that described by Bell in U.S. 4,485,096, the teachings of which are incorporated herein by reference.

Fibroblasts expressing the incorporated genetic material can also be introduced into the central nervous system. This can be done, for example, by introducing genetically engineered fibroblasts of the present invention (i.e., fibroblasts into which a gene of interest has been introduced) directly into specific regions of the brain through stereotactic administration. It can also be introduced into the cerebrospinal fluid via lumbar puncture or directly into the ventricles, which would result in seeding of the fibroblasts along the meninges.

Once introduced into or applied to the body, the transduced fibroblasts provide a continuous supply of the hormone, enzyme or drug encoded by the genetic material of interest. In the example described, the encoded product is hPTH.

The amount of the hormone, enzyme or drug supplied in this way can be modified or regulated as needed. This is done, for example, by using external cues or factors which affect their production; by controlling the size of the graft applied or the quantity of fibroblasts introduced into the body; or by removing the graft.

Cultured Fibroblasts

Fibroblasts are obtained from a subject by skin biopsy (e.g., a small punch biopsy from any area of

the body). The resulting tissue is placed in tissue culture media and separated into small pieces (e.g., by use of scalpels to tease apart the tissue). Small chunks of the tissue are placed on a wet surface of a tissue culture flask; approximately 10 pieces are placed in each flask. The flask is turned upside-down, closed tight and left at room temperature overnight. After 24 hours at room temperature, the flask is inverted; the chucks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media with 10% FBS and penicillin and streptomycin) is added. This is then incubated at 37^{o} for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerges. The monolayer is trypsinized and scaled up into larger flasks. The fibroblasts can be maintained in culture for approximately 50 generations; at approximately that time, they undergo what is called a crisis and subsequently do not grow very well. Soon after the fibroblasts are scaled up to (replated onto) larger flasks, they are infected according to the protocol described below.

Retroviral Vectors

Retroviruses are RNA viruses; that is, the viral genome is RNA. This genomic RNA is, however, reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. As shown in Figure 1,

the retroviral genome and the proviral DNA have three genes: the gag, the pol and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (nucleocapsid) proteins; the pol gene encodes the RNA-directed DNA polymerase (reverse transcriptase); and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of virion RNAs.

Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984).

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all virion proteins. Mulligan and co-workers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant stably integrated into the chromosome. Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed), 155-173 (1983); Mann, R., et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proceedings of the National Academy of

Sciences, U.S.A., 81:6349-6353 (1984). The teachings of these publications are incorporated herein by reference.

The Psi 2 cell line described by Mulligan and co-workers was created by transfecting NIH 3T3 fibroblasts with pMOV-Psi$^-$, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-Psi$^-$ expresses all the viral gene products but lacks the Psi sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi$^-$ expresses an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi am line, which are Psi-2-like packaging cell lines. These Psi-am cell lines contain a modified pMOV-Psi-genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology, 19:19-25 (1976). As a result, they are useful for production of recombinant virus with amphotropic host range. The retrovirus used to make the Psi am cell line has a very broad mammalian host range (an amphotropic host range) and can be used to infect human cells. If the recombinant genome has the Psi packaging sequence, the Psi-am cell line is capable of packaging recombinant retroviral genomes into infectious retroviral particles. Cone, R. and Mulligan, R., Proceedings of the National Academy of Sciences, USA, 81:6349-6353 (1984).

The retroviral genome has been modified by Cone and Mulligan for use as a vector capable of

introducing new genes into cells. As shown in Figure 2, the gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene serves as a dominant selectable marker. The retroviral sequence which remains part of the recombinant genome includes the LTRs, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984).

Additional vector constructions which have been used in producing transduced fibroblasts of the present invention are represented in Figure 2 and are described in detail below.

pZip. The construction of this vector has been described in Cepko, C.L. et al. Cell 37:1053 (1984). Briefly, this vector is capable of expressing two genes: the gene of interest and the Neo gene as a selectable marker.

The gene of interest is cloned into a BamHI site just distal to the 5' LTR, flanked by a splice donor site, and a splice acceptor site. Two transcipts will result from transcription of the provirus: the unprocessed transcript will result in expression of the gene of interest while the "processed" transcript will result in expression of the Neo gene.

pLJ. The characteristics of this vector have been described in Korman, A.J. et al., Proceedings of the National Academy of Sciences, USA 84:2150 (1987). This vector is capable of expressing two genes: the gene of interest and a dominant selectable marker, such as the Neo gene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning

site just distal to the 5' LTR, while, the Neo gene is placed distal to an internal promoter (from SV40) which is farther 3' than is the cloning site (is located 3' of the cloning site). Transcription from PLJ is initiated at two sites: 1) the 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the Neo gene.

pWe. The construction and initial characterization of this vector have been described. Choudory, P.V. et al, CSH Symposia Quantitative Biology, L.I. 1047 (1986). Briefly, this vector can drive expression of two genes: a dominant selectable marker, such as Neo, which is just downstream from the 5' LTR and a gene of interest which can be cloned into a BAMH1 site just downstream from an internal promoter capable of high level constitutive expression. Several different internal promoters, such as the beta-actin promoter from chicken (Choudory, P.V. et al, CSH Symposia Quantitative Biology, L.I. 1047 (1986)), and the histone H4 promoter from human (Hanly, S.M. et al., Molecular and Cellular Biology 5:380 (1985)) have been used. Expression of the Neo gene is from a transcript initiated at the 5' LTR; expression of the gene of interest is from a transcript initiated at the internal promoter.

pEm. In this simple vector, the entire coding sequence for gag, pol and env of the wild type virus is replaced with the gene of interest, which is the only gene expressed. The components of the pEm vector are described below. The 5' flanking

sequence, 5' LTR and 400 bp of contiguous sequence (up to the BAMHI site) is from pZIP. The 3' flanking sequence and LTR are also from pZIP; however, the cla site 150 bp upstream from the 3' LTR has been linkered with BAMH1 and forms the other half of the BamHI cloning site present in the vector. The HindIII/EcoR1 fragment of pBR322 forms the plasmid backbone. This vector is derived from sequences cloned from a strain of Moloney Murine Leukemia virus. An analogous vector has been constructed from sequences derived from the myeloproliferative sarcoma virus.

pIp. This vector is capable of expressing a single gene driven from an internal promoter. The construction of these vectors is summarized below. The 5' section of the vector, including the 5' flanking sequences, 5' LTR, and 1400 bp of contiguous sequence (up to the xho site in the gag region) is derived from wild type Moloney Leukemia virus sequence. Shinnick et al., Nature, 293:543 (1918). The difference between the two is that a SacII linker is cloned into an HaeIII restriction site immediately adjacent to the ATG of the gag gene. The 3' section of the vector, including the 3' flanking sequences, 3' LTR and 3' contiguous sequence (up to the claI site in the env coding region) is from pZIP. However, there are two modifications: 1) the claI site has been linked to BamHI and 2) a small sequence in the 3' LTR spanning the enhancer (from PvuII to XbaI) has been deleted. Bridging the 5' and 3' sections of the vector is one of several promoters; each one is contained on a xhoI/BamHI fragment, and

each is capable of high level constitutive expression in most tissues. These promoters include beta-actin from chicken (Choudory, P.V. et al, CSH Symposia Quantitative Biology, L.I. 1047 (1986), and thymidine Kinase from Herpes Simplex Virus, histone H4 from human (Hanly, S.M. et al., Molecular and Cellular Biology 5:380 (1985)). The vector backbone is the HindIII/EcoRI fragment from pBR322. The gene of interest is cloned into the BamHI site in direct orientation, just downstream from the internal promoter.

RO vectors. This category represents a heterogeneous group of vectors in which the gene of interest contains all the sequences necessary for transcription (i.e., promoter/enhancer, coding sequence with and without introns, and poly adenylation signal) and is introduced into the retroviral vector in an orientation in which its transcription is in a direction opposite to that of normal retroviral transcription. This makes it possible to include more of the cis-acting elements involved in the regulation of the introduced gene. Virtually, any of the above described genes can be adapted to be a RO vector. One example is described by Cone et al. in which the entire beta globin gene was cloned in reverse orientation into the BamHI site of pZip. Cone, R. and R.C. Mulligan Proceeding of the National Academy of Sciences, U.S.A., 81:6349-6353 (1984). RO vectors have been constructed in which the gene of interest has been cloned into the XhoI/BamHI site of pIp (essentially replacing the internal promoter).

Vectors into which a gene of interest has been inserted are shown in Table 1 as well as results obtained when the construction was tested for expression.

Table 1 Vectors Containing Gene of Interest

TABLE 1

Protein of Function	Vector					
	pZip	pLJ	pWe	pEm	pIP	pRO
Human growth homone	3	3				3
Human PTH	3	3		3	3	
Human receptor for LDL				3	3	
Human albumin		3	2			1
Human ornithine transcarbamlyase				1	1	
Human adenosine deaminase					3	
Receptor for interleukin-2			3	3		
Human Beta globin						3
Human alpha globin						3
Mutant dihydrofolate reductase	3			3	3	
Multidrug resistance					3	
Human glucose cerebrosidase			3			
Neomycin	3	3	3	3	3	
EIA gene from aderovirus	3					
Histidinol^{R} from *E. coli*	3					
-galactosidase from *E. coli*		3				
HLA antigens from human		3				
Human insulin		3				
Envelope from Moloney MLV	1		3			

[1] constructed, not tested for expression
[2] constructed, no expression when tested
[3] constructed, expression detected

Vectors which include a selectable marker are represented in Figure 2a, 2b and 2c. In Figure 2a, pZIP neo SVX, described by Cepko is represented. Cepko, C. et al., Cell, 37:1053-1062 (1984). Here, there is a splice donor and splice accept, noted as SD and SA, between which the gene of interest is inserted, using known techniques. Expression of the inserted gene is based on the LTR in the unprocessed message; the processed message is responsible for expressing neomycin.

In Figure 2b, vector pLJ is represented. In pLJ, the genetic material of interest is inserted just following the 5' LTR. Expression of this genetic material is transcribed from the LTR and expression of the neo gene is transcribed from an internal SV40 promoter.

In Figure 2c, the pWe vectors are represented. In pWe vectors, the LTR promoter and transcript is responsible for expressing neomycin and an internal promoter is responsible for expression of the gene of interest. Promoters useful in this type of vector include promoters derived from chicken beta-actin, human histone, Herpes simplex thymidine kinase, thy 1 (a tissue-specific promoter used in T cells) and rat albumin.

Vectors without a selectable marker can also be used to transduce fibroblasts with genetic material of interest. Such vectors are basically simplifications of the vectors previously described, in which there is such a marker. Vector pEm is represented in Figure 2d; as represented, the main components of the vector are the 5' and 3' LTR, and the genetic

material of interest, inserted between the two LTRs. pIp represents a series of useful vectors in which there is an internal promoter. As represented in Figure 2e, there is an LTR at each end, flanking an internal promoter; promoters useful in these vectors include those derived from chicken beta-actin, human histone, Herpes simplex thymidine kinase and rat thy 1.

Introduction of Genetic Material into Fibroblasts and Assessment of Expression of the Genetic Material

A cell line which produces recombinant amphotropic retrovirus having a recombinant genome is used to infect fibroblasts. As described above, the recombinant genome can include a variety of components, but in general is comprised of two LTRs and, in place of the gag, the pol and the env sequences, a second promoter sequence and, in some cases, a gene encoding a selectable marker (e.g., neo).

Viral stocks, to be used in introducing genetic material of interest into fibroblasts, are harvested, as described above, supplemented with 8 micrograms per ml. (mcg/ml.) of Polybrene (Aldrich) and added to the culture of fibroblasts. If the titer of the virus is high (e.g., approximately 10^6 cfu per ml.), then virtually all fibroblasts will be infected and no selection (e.g., of fibroblasts into which the vector, including the recombinant genome, has been introduced) is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. If a

selectable marker is used, after exposure to the virus, the cells are grown to confluence and split into selective media (e.g., media containing G418 if the selectable marker is neo, media containing histidinol and no histidine if the selectable marker is his).

The neo gene is a bacterial gene derived from the transposon Tn5, which encodes neomycin resistance in bacteria and resistance to the antibiotic G418 in mammalian cells. This neo gene acts as a dominant selectable marker; its presence in a mammalian cell converts the cell into one which will grow in the presence of G418. (If it is not present, the cell dies in the presence of G418.) As a result, the presence of this gene in a mammalian cell can be determined by culturing cells in media which contains G418. The recombinant retrovirus having this recombinant genome is referred to as the neo virus.

The recombinant retroviral vectors having the neo gene also have a cloning site. As a result, genetic material of interest can be introduced into the vector, incorporated into fibroblasts and expressed by fibroblasts transduced with the recombinant retrovirus (referred to as fibroblasts having incorporated genetic material).

At the BamHI cloning site, it is possible to insert genetic material of interest. The genetic material of interest can be DNA which does not normally occur in fibroblasts; DNA which normally occurs in fibroblasts but is not expressed by them at levels which are biologically effective (i.e., levels sufficient to produce the normal physiological

effects of the polypeptide it encodes); DNA which occurs in fibroblasts and has been modified so that it is expressed by such cells; and any DNA which can be modified to be expressed by fibroblasts, alone or in any combination thereof.

A copy of the gene encoding human parathyroid hormone (hPTH) has been cloned into this site, (e.g., into pLJ) in the following way: The pLJ plasmid was digested with BamHI and subsequently treated with the enzyme calf intestinal phosphatase. Following this, the linear vector was fractionated on argarose gel and purified, using glass beads. In addition, the BamHI fragment containing the human PTH gene was prepared from the plasmid described by Hendy et al., which contains a complete cDNA of human PTH cloned into pBR322. Hendy, G.N. et al., Proceedings of the National Academy of Sciences, USA, 78:7365-7369 (1981).

A sub fragment of the PTH cDNA, containing 17 bp of 5' untranslated, all coding and 31 bp of 3' untranslated sequence, was isolated by digesting the initial plasmid with DdeI and HinfI and isolating the 600bp fragment. The ends of this fragment were treated with DNA polymerase, to fill in the recessed ends. BamHI linkers were ligated to the blunt ends with T_4 DNA ligase. An authentic BamHI restriction fragment was generated by digesting the ligation mixture from above with BamHI. This was then subcloned into the BamHI site of pBR322, which is the plasmid used as the source of hPTH in vector construction.